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This proposal is designed to investigate the role of PTHrP in breast cancer-mediated osteolysis. Observations in patients with bone metastases suggest that breast cancer cells in bone express PTHrP more frequently than in soft tissue sites of metastasis or in the primary tumor. Three isoforms of PTHrP, 1-139, 1-141 and 1-173, are products of alternative splicing in humans, but the specific contribution of each of these isoforms to osteolytic metastasis caused by breast cancer has not been evaluated. To determine the role of these isoforms in breast cancer metastasis to bone, the human breast cancer cell line MDA-MB-231 (MDA-231) was stably transfected with similar amounts of cDNAs for human prepro PTHrP-(1-139), -(1-141) or -(1-173), driven by a CMV promoter, and studied in a model of human breast cancer metastasis to bone. Conditioned media from stable MDA/PTHrP-(1-139) clones contained significantly more PTHrP, compared with MDA/PTHrP-(1-141), -(1-173) or parental MDA-231. Nude mice were inoculated into the left cardiac ventricle with MDA-231/ PTHrP-(1-139), -(1-141), -(1-173) clones or parental MDA-231 cells. Osteolytic lesion area of radiographs was greatest in mice bearing MDA/PTHrP-(1-139) compared with those bearing MDA/PTHrP-(1-141), -(1-173) or parental MDA-231. Ca^{++} was significantly higher in the MDA/PTHrP-(1-139) compared with the MDA/PTHrP-(1-141), -(1-173) or parental MDA-231 groups as was the plasma PTHrP concentration. The data demonstrate that overexpression of PTHrP-(1-139) isoform in the human breast cancer cell line MDA-MB-231 results in greater PTHrP secretion *in vitro* and enhanced osteolysis with increased plasma PTHrP concentrations and hypercalcemia *in vivo* compared with overexpression of PTHrP-(1-141) or -(1-173). Differential cell processing of the isoforms may result in more efficient secretion of PTHrP-(1-139) and the osteolysis that is characteristic of breast cancer

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Theresa A Gunn 10/27/97

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INTRODUCTION

NATURE OF THE PROBLEM AND BACKGROUND OF PREVIOUS WORK

Breast Cancer, Hypercalcemia and Osteolysis

Breast cancer is associated with significant morbidity in the skeleton. Specifically, breast cancer can involve bone through both metastatic and humoral mechanisms. Metastases to bone are more commonly osteolytic than osteoblastic and are responsible for the complications of bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes that many breast cancer patients suffer from (1). Eighty-four per cent of patients dying of breast cancer have bone metastases (2).

Hypercalcemia is commonly associated with breast cancer, occurring in up to 40% of afflicted women during the course of their disease (2,3). Skeletal destruction by metastatic tumor has been felt to be the major mechanism responsible for hypercalcemia (3). Increased osteoclastic bone resorption in areas surrounding breast cancer metastasis has been documented histologically (4,5) suggesting that factors secreted by breast cancer cells can locally activate osteoclasts. Recent evidence, however, suggests that osteolytic bone metastasis may not be the only mechanism responsible for breast cancer hypercalcemia and that humoral mechanisms may contribute in as much as 30-60% of the cases (6-8). In one study, 15% of 147 hypercalcemic breast cancer patients had no bone metastases (9).

PTHrP and Breast Cancer

Parathyroid hormone-related protein (PTHrP) is a major mediator of humoral hypercalcemia of malignancy, due to its PTH-like actions. This protein was purified in 1987 from human lung cancer (10), breast cancer (11) and renal cell carcinoma (12) simultaneously by several independent groups. Cloning and expression followed shortly thereafter (13).

PTHrP has since been extensively studied and found to have many similarities to PTH. It has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (13), binds to PTH receptors (14) and shares similar biologic activity to PTH (15). Specifically, it stimulates adenylate cyclase in renal and bone systems (11,12,15-17), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (16,17), decreases renal phosphate uptake (15,16,18) and stimulates 1α-hydroxylase (15). PTHrP has been found in a variety of tumor types as well as normal tissue (19-22). The widespread expression of PTHrP in normal as well as malignant tissue was the first evidence that the hormone has a role in normal physiology. In addition to the PTH-like effects, PTHrP has many non-PTH-like properties (23), some of which include regulation of placental calcium transport (22), possible establishment of bone metastasis in breast cancer (24,25), and autocrine regulation of the growth of some tumors (26). The regulation of PTHrP is poorly understood, but factors such as prolactin (27), glucocorticoids, $1,25(OH)_2D_3$ (28), epidermal growth factor (28), TGFα (29), TGFβ (30), estrogen (31-34) and stretch (35) have been shown to regulate gene expression and extracellular calcium concentration has been shown to control the production of PTHrP in vitro in Leydig tumor cells (36).

It is now clear that PTHrP is a significant factor in mediating hypercalcemia in breast

cancer (37). One of the 3 tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (11). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (24). By immunohistochemistry (25) and in situ hybridization (38), it was detected in 12 of 13 breast cancer metastases in bone prompting speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastasis. Along these lines, Bundred and colleagues found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women and PTHrP positivity was related to the development of bone metastases (39). Additionally, 65-92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by radioimmunoassay (RIA) similar to those documented in patients with humoral hypercalcemia of malignancy due to non-breast tumors (40,41).

PTHrP in Nonmalignant Breast Disease

In addition to its role in malignancy, PTHrP is important in the normal physiology of breast (42). It is expressed in lactating mammary tissue (43) and secreted into milk at concentrations 10,000-100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (44-48). Suckling increases PTHrP gene expression and this appears to be mediated through prolactin (49). Estrogen has been shown to increase PTHrP expression in uterine tissue and *in vitro* studies suggest that there may be estrogen response elements present in the PTHrP gene (50-53). Increased plasma PTHrP concentrations have been described in at least 2 patients with the rare syndrome of lactational hypercalcemia (54-56). Animal studies have demonstrated a PTHrP gradient across the mammary gland in lactating goats (48) indicating that PTHrP may gain access to the maternal circulation during lactation. In support of this, a recent clinical study has shown detectable plasma PTHrP concentrations in 63% of breast-feeding mothers while similar measurements in bottle-feeding control mothers were undetectable (57). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production and it may be the implicating factor in lactation-associated bone loss (58).

PTHrP as a Growth Regulator

PTHrP is produced in relatively low concentrations in breast myoepithelial cells (59). A transgenic mouse model, in which PTHrP is over expressed in skin and breast myoepithelial cells through the use of a human keratin promoter, has demonstrated breast hypoplasia. Specifically, female transgenics had a severe reduction in the number of albeit normal terminal ducts and acini in the breast suggesting that PTHrP may play a role in regulating ductular proliferation and/or differentiation during mammogenesis (60). These mice also had failure of normal hair follicle development indicating a similar role for PTHrP in the skin.

Along those lines, disruption of PTHrP expression in a normal keratinocyte cell line, using antisense technology, results in enhanced growth of the cells in culture (61). *In vivo*, homozygous mice for the PTHrP null mutation are born with a multitude of skeletal abnormalities, including defects in the bone growth plate (62). These findings, along with those of the above described transgenic mice, suggest that either over- or under- expression of PTHrP in normal cells result in abnormalities of growth and possibly differentiation.

In malignant cells, PTHrP has been shown to act as an autocrine growth factor in a renal cell carcinoma cell line (26) and more recently, in a squamous cell carcinoma line (63). There are no reported studies on the role of PTHrP as an autocrine growth factor in breast cancer.

Regulation of PTHrP by Other Tumor-associated and Bone-derived Growth Factors

Other tumor-associated growth factors as well as bone-derived growth factors may be important regulators of PTHrP expression in both malignant and non-malignant tissue. Epidermal growth factor has been shown to increase PTHrP expression in a keratinocyte cell (64) line while TGF-α, a breast cancer tumor product (65), enhances PTHrP expression in a human squamous cell carcinoma of the lung (29). Moreover, other tumor-associated factors may modulate the end organ effects of PTHrP. TGF-α enhances the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (66) and it can modulate the renal and bone effects of PTHrP as well (67,68). Additionally, TGF-β, which is present in high concentrations in the bone microenvironment, has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (30) as well as in an epidermal squamous cell carcinoma (69).

Implications of PTHrP Status in Breast Cancer

These findings have important implications for the ability of breast cancer to affect the skeleton. First, breast cancers expressing PTHrP in addition to other tumor-associated factors, such as TGF-α (65), may be more likely to affect the skeleton through humoral and osteolytic mechanisms if the co-expressed factor enhances PTHrP expression in the primary tumor. Second, if estrogen regulates PTHrP expression in breast cancer cells as it does in other tissues, estrogen receptor positive tumors may preferentially express PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP. TGF-β, as well as other bone derived growth factors, are present in high concentration in the bone microenvironment (70) and are released from bone during the process of osteoclastic bone resorption (71). PTHrP expression in breast cancer cells lodged in bone is likely to be increased in the presence of TGF-β. In this scenario, osteoclastic bone resorption is increased further causing release of more TGF-β and other growth factors into the bone microenvironment leading to further enhancement of PTHrP expression in the breast cancer cells. If PTHrP acts as an autocrine growth factor in breast cancer cells, as it does in some tumor models, then tumor growth would be enhanced as well. The clinical findings of an increased incidence of PTHrP expression in bone compared with other sites by Powell and colleagues (25,38) supports the notion that production of PTHrP as a bone resorbing agent may contribute to the ability of breast cancers to grow as bone metastases.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize to bone due to its potent bone resorbing capability, early treatment with inhibitors of bone resorption is likely to prevent or delay the development of bone metastases as well as reduce the catastrophic complications of pain, hypercalcemia, fracture and nerve compression syndromes. It is already clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in advanced breast cancer (72-74). Bisphosphonates have also been shown to decrease the number of bone metastases in animal models (75,76), but it is unclear whether or not these tumors express PTHrP. However, since the safety of long term bisphosphonate use has not been determined and bone mineralization defects can occur with high doses of these drugs, it would be of benefit, as well as cost effective, to identify which patients are at risk to develop bone metastases and treat only those rather than treat all women with breast cancer. The clinical evidence thus far supports PTHrP as a marker to identify such women, but better animal models are needed to clarify this role.

Knowledge of PTHrP status may also have significant therapeutic implications in treating breast cancer-associated hypercalcemia. Although hypercalcemia in breast cancer is often associated with bone metastases, it is clear that humoral mechanisms may contribute in as much as 60% of the cases. Traditionally, treatment has been directed toward inhibiting bone resorption and this is often effective. However, it has now become evident that bisphosphonate therapy is less effective in patients with higher plasma concentrations of PTHrP and without radiological evidence of bone metastases (77,78). Thus, inhibition of bone resorption is effective when the major mechanism for hypercalcemia is increased bone resorption. Since PTHrP causes hypercalcemia by both increasing osteoclastic bone resorption and increasing renal tubular reabsorption of calcium, drugs that inhibit bone resorption alone may not normalize the calcium concentration if the plasma PTHrP concentration is high enough to add a significant renal component to the hypercalcemia. Drugs directed against either the actions of PTHrP or the secretion of PTHrP may therefore be more beneficial in the bisphosphonate resistant situation. Unfortunately, no such drugs are available at the current time but the need for them is obvious. A potentially useful therapy may prove to be the use of monoclonal antibodies against PTHrP. Sato has recently described successful use of an anti-PTHrP-(1-34) monoclonal murine antibody in an animal model of humoral hypercalcemia that ameliorated hypercalcemia and prolonged survival time in severely ill animals (79).

In Vivo Models of Hypercalcemia and Osteolysis

These observations demonstrate the need for further study of the role of PTHrP in malignant and nonmalignant breast disease. The only research done to date on PTHrP expression in human breast cancer and its potential role in humoral hypercalcemia and the development of osteolytic bone metastases have involved the small clinical studies described above (24,25,39-41). Despite numerous animal models of human breast cancer (80) that have been described to date, human breast cancer cell lines have not been studied *in vivo* for PTHrP expression and its relationship to the development of osteolytic bone metastases and humoral hypercalcemia. Most animal models of breast cancer have been used to evaluate the effect of various factors (81-83) on breast cancer growth. Only one spontaneous rat mammary tumor (Walker 256 carcinosarcoma) has been shown to cause humoral hypercalcemia in rats (84), produce PTHrP (85) and cause osteolytic bone metastases (75). Given the accumulating evidence documenting a humoral mechanism for hypercalcemia in breast cancer, the established role of PTHrP in humoral hypercalcemia of malignancy, the presence of PTHrP in malignant as well as lactating breast tissue and the presence of PTHrP in established breast cancer metastases to bone, it is evident that established models of human breast cancer should be evaluated for PTHrP expression and its relationship to the skeleton. Using animal models will be beneficial in defining this aspect of the pathophysiology of breast cancer and this will in turn have important prognostic and therapeutic implications.

Historically, it has been difficult to produce bone metastases in animal models of malignancy. Tumors inoculated subcutaneously or intramuscularly do not metastasize in nude mice and tumors inoculated into the tail vein usually produce only lung metastases. Yoneda has developed an animal model of human breast cancer cell metastasis to bone (76,86) which is based on a model originally described by Arguello (87). In this model, MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. This model has been used to show that the bisphosphonate, risedronate, decreased osteolytic lesions when given simultaneously with tumor cells and inhibited both an increase in new bone metastases and progression of each metastatic focus when give to animals with pre-existing osteolytic lesions (76).

PURPOSE OF PRESENT WORK

Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP is an important humoral mediator of hypercalcemia in cancer and may have physiologic roles in the lactating breast as well as in cell growth and differentiation. The role of PTHrP in the pathophysiology of breast cancer is significant for several reasons. 1) PTHrP mediates hypercalcemia through its systemic effects of increasing osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. 2) Due to its potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are so characteristic of patients with breast cancer. 3) Growth factors present in the bone microenvironment further enhance PTHrP expression in breast cancer cells present in bone and promote development of osteolytic lesions and tumor growth. Thus, PTHrP expression in the primary breast tumor may be a marker for the development of hypercalcemia and bone metastases.

The purpose of this study is to define the role of PTHrP in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. Our previous studies reported in the first 2 years of this proposal indicate that 50% of breast cancer cell lines tested secrete low, but significant amounts of PTHrP. Over-expression of PTHrP-(1-141) increased osteolytic metastasis in a mouse model of human breast cancer metastasis to bone. Furthermore, treating mice with a neutralizing antibody to PTHrP inhibited the development of new bone metastasis and the progression of established bone metastasis caused by MDA-MB-231, a human breast cancer cell line which makes low amounts of PTHrP. Since it is now clear that PTHrP has an important role in the development and progression of breast cancer metastasis to bone, our aim was to investigate the role of the various isoforms of PTHrP in the pathophysiology of breast cancer metastasis to bone.

The human PTHrP gene is complex and spans approximately 15 kilobases of genomic DNA and is composed of nine exons (99). Three isoforms of PTHrP, 1-139, 1-141 and 1-173, are products of alternative splicing in humans and depend on whether exon VI is spliced to exon VII, VIII, or IX respectively. The gene is under the control of three distinct promoters and agents which regulate PTHrP expression such as transforming growth factor β (TGFβ), glucocorticoids and epidermal growth factor act al least in part by altering the rate of gene transcription (99). However, the specific contribution of each PTHrP isoform to osteolytic metastasis caused by breast cancer or hypercalcemia is not known.

To determine the role of these isoforms in breast cancer metastasis to bone, the human breast cancer cell line MDA-MB-231 (MDA-231) was stably transfected with similar amounts of cDNAs for human prepro PTHrP-(1-139), -(1-141) or -(1-173), driven by a CMV promoter, and studied in a model of human breast cancer metastasis to bone. The results presented here demonstrate that although breast cancer cells expressing the PTHrP-(1-139) isoform had similar in vitro growth rates as those expressing the other isoforms or the parental MDA-MB-231 cells, PTHrP secretion was markedly increased. This was associated with enhanced osteolysis and hypercalcemia when the cells were studied in a mouse model of human breast cancer metastasis to bone. Information gained from these studies will have important prognostic and therapeutic implications.

METHODS OF APPROACH

In order to define the role of PTHrP in the pathophysiology of breast cancer-associated hypercalcemia and skeletal complications in a systematic fashion, the following objectives were originally proposed.

1. **SPECIFIC AIM #1: To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.**

    a. Known breast cancer cell lines (both estrogen receptor positive and negative) will be grown in culture along with positive and negative controls. Media conditioned for 24 hours will be screened for PTHrP immunoreactivity by immunoradiometric assay.

    b. RNA will be isolated from above cell lines in the presence and absence of estrogen and PTHrP expression will be determined using Northern analysis.

2. **SPECIFIC AIM #2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.**

    a. Measure standard parameters of calcium homeostasis in nude mice bearing human breast tumors.

    b. Determine that hypercalcemia observed in mice bearing PTHrP+ breast tumors is PTHrP-mediated. Two approaches will be used: i) to decrease PTHrP secretion by transfecting PTHrP + lines with PTHrP antisense ii) decrease PTHrP effects by administration of neutralizing antibody.

        1. Transfection of PTHrP antisense cDNA into breast cancer cell lines that secrete PTHrP and cause hypercalcemia in nude mice.

        2. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that have been transfected with PTHrP antisense cDNA.

        3. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that are treated with anti-PTHrP-(1-34) monoclonal antibody.

3. **SPECIFIC AIM #3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.**

a. Is PTHrP expression enhanced in the bone microenvironment relative to other metastatic sites? Using an animal model of breast cancer-mediated osteolysis, PTHrP expression will be compared in bone and non-bone sites using immunohistochemistry and in situ hybridization.

b. Does expression of PTHrP in the primary tumor enhance the development and quantity of osteolytic bone metastases? Breast cancer cell line, MDA-231 will be transfected with the cDNA for human PTHrP or PTHrP-AS (antisense orientation as a control) and used in the osteolytic model.

1. Production of stable MDA-231 clones expressing PTHrP or PTHrP-AS by calcium phosphate precipitation.

2. Effect of MDA-231/PTHrP on development of osteolytic bone metastases will be assessed by inoculating these cells into the left ventricle of mice and determining if the quantity and size of the bone metastases differ from similarly inoculated control MDA-231/PTHrP-AS. Neutralizing antibodies will be given to attempt to block osteolysis in mice inoculated with MDA-231/PTHrP cells.

BODY

METHODS

Cell culture

MDA-MB-231 cells (13) were (provided by C. Kent Osborne, M.D.) were cultured in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Hyclone, Logan, UT), 1% penicillin/streptomycin and nonessential amino acids (Gibco, Gaithersburg, MD) in a 37°C atmosphere of 5% CO_2/air. To test the effect of TGFβ on PTHrP secretion by MDA-MB-231 cells, 10^4 cells/mL were plated onto 48 well plates. When near confluence, cells were washed with phosphate buffered saline (PBS) and 250 µl of serum-free DMEM containing TGFβ1 (5 ng/mL) was added to each well. TGFβ1 was purchased from R & D, Minneapolis, MN. Conditioned media were collected after 48 hours and stored at -70°C for PTHrP measurement. Cell number was counted for each well to correct the PTHrP concentration of the conditioned media. Triplicate measurements were performed.

To determine the growth rate of MDA-MB-231 cells and respective clones expressing the PTHrP-(1-139), -(1-141) or -(1-173) isoforms, 10^4 cells/mL were plated onto each of 24-well plates. Cell number was counted every day for eight days and each measurement was performed in triplicate.

Stable transfection of MDA-MB-231 cells with cDNA for human prepro PTHrP-(1-139), -(1-141) or -(1-173)

The pcDNA3/PTHrP-(1-139), -(1-141), -(1-173) or the empty vector pcDNA3 was transfected into MDA-MB-231 cells by calcium phosphate precipitation (Sambrook). Single clones were isolated by limiting dilution in the presence of the selective marker, G418 (Sigma, St. Louis, MO). Clones were screened by measuring the amount of secreted PTHrP in serum-free 48-hour conditioned media.

***In vivo* experiments**

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice 4-6 weeks of age were housed in laminar flow isolated hoods with 12 hour light/12 hour dark cycle. Water supplemented with vitamin K and autoclaved mouse chow were provided ad libitum.

Whole blood samples for ionized calcium concentration were obtained by retro-orbital puncture under metofane anesthesia. Blood samples for PTHrP measurement were similarly obtained and collected on ice in vacutainer tubes containing EDTA (Becton Dickinson, NJ) and 400 IU/mL aprotinin (Sigma, St. Louis, MO).

Tumor inoculation into the left cardiac ventricle was performed while the mice were anesthetized with a ketamine/xylazine mixture and positioned ventral side up based on a modification of Arguello (87). The left cardiac ventricle was punctured percutaneously using a 27 gauge needle attached to a 1 mL syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicated correct position in the left cardiac ventricle.

Experimental protocols

Bone metastasis

Mice were inoculated with tumor cell suspensions of MDA/PTHrP-(1-139), MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) or MDA/pcDNA3 cells into the left cardiac ventricle (n=7 per group) on day 0. Baseline radiographs and body weights as well as blood for Ca^{2+} and plasma PTHrP concentrations were obtained at this time. Radiographs were taken on day 21 and then weekly until sacrifice to monitor progression of osteolytic metastasis. Ca^{2+} and body weight were measured weekly for three weeks post tumor inoculation until sacrifice, at which time most mice

in the control groups were cachectic and paraplegic. At the time of sacrifice, blood was collected for Ca^{2+} and PTHrP measurement, and all bones and soft tissues were harvested and fixed in formalin for histologic analysis. Autopsy was performed on all mice, and those with tumor in the chest were excluded from analysis, as this indicated that part or all of the tumor inoculum did not properly enter the left cardiac ventricle.

Analytical Methods

Ca^{2+} measurement
Ca^{2+} concentrations were measured in whole blood using a Ciba Corning 634 ISE Ca^{2+}/pH analyzer (Medfield, MA) and adjusted using the internal algorithm of the instrument to pH 7.4. Samples were run in duplicate and the mean value recorded.

PTHrP Assay
PTHrP concentrations were measured in conditioned media and plasma using a 2-site immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA) which uses 2 polyclonal antibodies that are specific for the N-terminal -(1-40) and -(60-72) portions of PTHrP and has a calculated sensitivity of 0.3 pmol/L (90). PTHrP concentrations in conditioned media samples were calculated from a standard curve generated by adding recombinant PTHrP-(1-86) to the specific type of medium (unconditioned) used and were considered undetectable if media concentrations were <0.3 pmol/L prior to correction for cell number.

Radiographs and measurement of osteolytic lesion area
Animals were x-rayed in a prone position against the film (22 x 27cm X-Omat AR, Kodak) and exposed with x-rays at 35 KVP for 6 seconds using a Cabinet X-ray system-Faxitron Series, Hewlett-Packard (Model 43855A), (Faxitron X-ray Corporation, Buffalo Grove, IL). All radiographs were evaluated in blinded fashion. The area of osteolytic bone metastases was calculated using a computerized image analysis system. Video images of radiographs were captured using a frame grabber board (Targa+, Truevision, Inc., USA) on a PC system. Quantitation of lesion area was performed using image analysis software (Java, Jandal Video analysis, Jandel Scientific, CA).

Statistical analysis
Results are expressed as the mean ± the standard error of the mean. Data were analyzed by repeated measures analysis of variance followed by Tukey-Kramer post test. P values of <0.05 were considered significant.

RESULTS

Stable clones expressing the cDNA for the human preproPTHrP-(1-139), -(1-141), or (1-173) secreted different amounts of PTHrP as detected by IRMA of serum-free conditioned media (Figure 1). All transfectants secreted significantly more PTHrP than the parental MDA-MB-231 cells. Those MDA-MB-231 cells expressing PTHrP-(1-139) consistently secreted the most PTHrP compared with the other isoforms. MDA-MB-231 cells expressing the PTHrP-(1-173) isoform secreted more PTHrP than those expressing the PTHrP -(1-141) isoform. This pattern of secretion was similar in transient transfections of 293 cells and suggests differential processing of the 1-139 isoform. All transfectants, except those expression the 1-139 isoform responded to TGFβ with an increase in PTHrP secretion. *In vitro* growth rates were similar for all transfectants and did not differ from the parental MDA-MB-231 cells (Figure 2).

In vivo, bone metastases developed in all groups tested: parental MDA-MB-231, PTHrP-(1-139), -(1-141) and -(1-173). Mice bearing the MDA/PTHrP-(1-139) developed strikingly larger bone metastases which occurred much earlier than those of mice bearing the MDA/PTHrP-(1-173) or -(1-141) (Figure 3). These differences were statistically significant as quantitated by computerized image analysis of radiographs (Fiugre 4). The total lesion area on radiographs was significantly larger in the mice bearing MDA/PTHrP-(1-139) compared with mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141). The latter 3 groups did not differ significantly with regard to lesion area. Mice bearing MDA/PTHrP-(1-139) had more lesions at day 48 compared with mice in the other groups (Fiugre 4). The lesion number in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141) reached values comparable to those of the MDA/PTHrP-(1-139) group, however, this was significantly longer after tumor inoculation.

Significant hypercalcemia was evident in mice bearing MDA/PTHrP-(1-139) tumors compared with those bearing the -(1-173), -(1-141) or parental MDA-MB-231 cells (Figure 5). This was due to a marked increase in the plasma PTHrP concentration in the MDA/PTHrP-(1-139) group determined at sacrifice. The PTHrP concentrations were similar to those observed in humans with malignancy-associated hypercalcemia (Burtis). Mice in all other groups remained normocalcemic throughout the experimental period. Plasma PTHrP concentrations were significantly higher in the mice bearing MDA/PTHrP-(1-173) and -(1-141) at the time of sacrifice when compared with those taken at baseline. However, these concentrations were not increased to the degree with which systemic effects of PTHrP should be observed. Additionally mice bearing the MDA/PTHrP-(1-139) tumors lost significantly more body weight than mice in the other groups.

In these experiments, metastasis to sites other than bone included adrenal gland, ovary, lung and liver in all groups. However, there were no significant differences in metastases to such nonbone sites between any of the groups.

DISCUSSION

The data presented here demonstrate that overexpression of PTHrP-(1-139) in the human breast cancer cell line, MDA-MB-231, is associated with enhanced PTHrP secretion *in vitro* compared with other isoforms of PTHrP-(1-173) and -(1-141). This enhanced PTHrP secretion was also evident *in vivo* as mice bearing the MDA/PTHrP-(1-139) tumors had increased plasma PTHrP concentrations that were significantly different from those at baseline or those at sacrifice in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) or MDA/PTHrP-(1-141). The enhanced PTHrP secretion by MDA-MB-231 cells *in vitro* and *in vivo* correlated to increased bone destruction and hypercalcemia in a mouse model of human breast cancer metastasis to bone.

These findings are consistent with the previous clinical and experimental evidence which implicate PTHrP as a mediator of the local bone destruction associated with breast cancer metastasis to bone. PTHrP is present in tumor cells in bone in the majority of patients with advanced breast cancer (25,38), and development of subsequent bone metastases is positively correlated with PTHrP expression in the primary site (39,40). Moreover, neutralizing antibodies to PTHrP abrogate the osteolytic bone lesions in a mouse model of human breast cancer metastases to bone (98). Finally, treatment with bisphosphonates, potent inhibitors of osteoclastic bone resorption are associated with decreased morbidity in patients with breast cancer metastases to bone (100).

Current evidence suggests that PTHrP expression by human breast cancer cells is enhanced in the bone microenvironment (25). Growth factors released in active form when bone is resorbed such as transforming growth factor β (TGFβ) (101) may enhance PTHrP production in that site. In these experiments parental MDA-MB-231 cells or those expressing the -(1-141) or -(1-173) isoforms significantly increased PTHrP production in response to TGFβ. Since the expression of the cDNAs are driven by a constitutive promoter, it is likely that the effects of TGFβ are post-transcriptional. One known effect of TGFβ is to stabilize the messenger RNA for PTHrP and this has been demonstrated in a renal cell carcinoma (102). The MDA-MB-231 cells expressing the PTHrP-(1-139) isoform did not respond to TGFβ by increasing PTHrP production. The reasons for this consistent finding are not clear but may be due to the possibility that the MDA/PTHrP-(1-139) cells are secreting the maximal amount of PTHrP and cannot be further stimulated.

The exact role of the different isoforms of PTHrP in cancer and in normal physiology are not known. Additionally, whether any isoform predominates in malignancy is unknown. Previous work has demonstrated that all isoforms are present in the malignant cell lines BEN, COLO 16, HcCaT, MCF7, MDA-MB-231 and T-47D at the mRNA level and that expression is cell-specific with regulator-induced promoter usage (99). What is clear from the work presented here is that overexpression of the PTHrP-(1-139) isoform results in more efficient secretion of PTHrP from MDA-MB-231 cells as well as from a small number of other cell lines which have been tested such as HEK 293 cells and the human breast cancer cell line, MCF7. This efficient secretion of PTHrP results in the enhanced osteolysis and hypercalcemia observed in this mouse model of bone metastases. Whether this enhanced capacity to cause bone metastasis is an isoform-specific property rather than due to an absolute increase in PTHrP production alone cannot be determined from these studies.

Another consideration is that the different PTHrP isoform proteins are processed into different peptides which have different actions. Like PTH and other endocrine peptides, PTHrP undergoes endoproteolytic posttranslational processing that results in several secretory forms: 1) an amino-terminal PTHrP-(1-36), 2) a mid-region species that begins at amino acid 38 that

has an undefined carboxyl terminus (104,105) and 3) a carboxyl-terminal species that is recognized by an antibody directed against the 109-138 region (104-106). The preponderance of and arrangement of basic residues in the protein sequence suggest that members of the subtilisin family of endoproteases such as furin (105), PC 1/3, PC-2, PACE-4 and PC8 (106) are responsible for such processing (107,108). Posttranslational modification of PTHrP also occurs as glycosylation of an amino-terminal PTHrP species produced by keratinocytes has been reported (109). Regulation of PTHrP secretion may be cell-specific as PTHrP expressed in neuroendocrine cells is secreted in a regulated fashion as compared with a constitutive secretion when expressed in non-neuroendocrine cell types such as squamous cell carcinoma (108). Although PTHrP mediates its calcemic effects through the classical PTH/PTHrP receptor, there is evidence for a separate PTHrP receptor (110). However, the function of such a receptor remains unclear.

In this study, PTHrP concentrations were measured *in vitro* and *in vivo* using a 2-site immunoradiometric assay which detects PTHrP-(1-72). Thus, it is not clear exactly which processed forms of PTHrP are secreted by the respective transfectants. However, these results suggest that differential cell processing of the isoforms may result in more efficient secretion of PTHrP-(1-139) and the consequent osteolysis that is characteristic of breast cancer.

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FIGURE 1

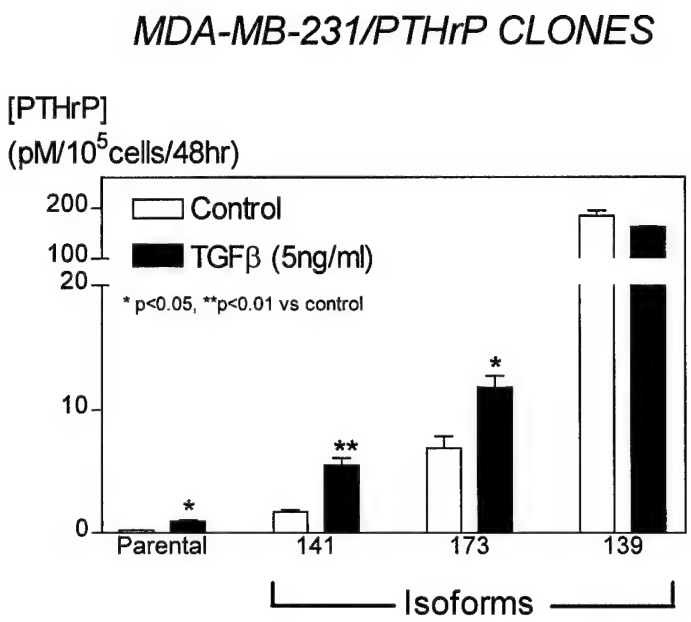


FIGURE 1: PTHrP secretion by parental MDA-MB-231 cells and representative clones MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) in the basal state and in response to TGFβ. Respective cells were plated onto 48-well plates and grown to near confluence. Cells were washed and incubated with serum-free media in the presence or absence of TGFβ (5 ng/mL) for 48 hours. PTHrP concentrations in conditioned media were corrected for cell number. Values represent the mean ± SEM. N = 3 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 2

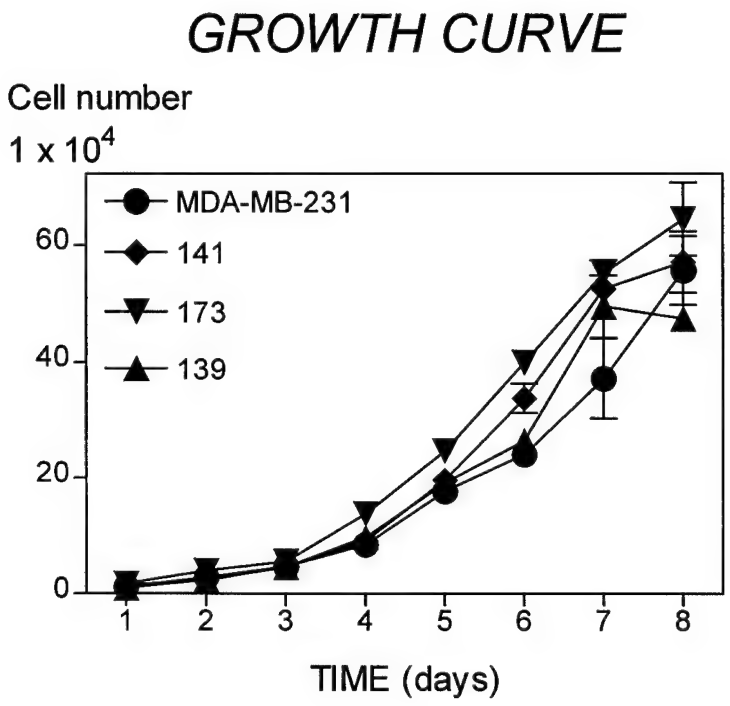


FIGURE 2: Growth rate of parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) cells. Respective cells were plated at a density of 10^4 cells/well in 10% FCS and counted daily. Values represent the mean ± SEM. N = 3 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 3

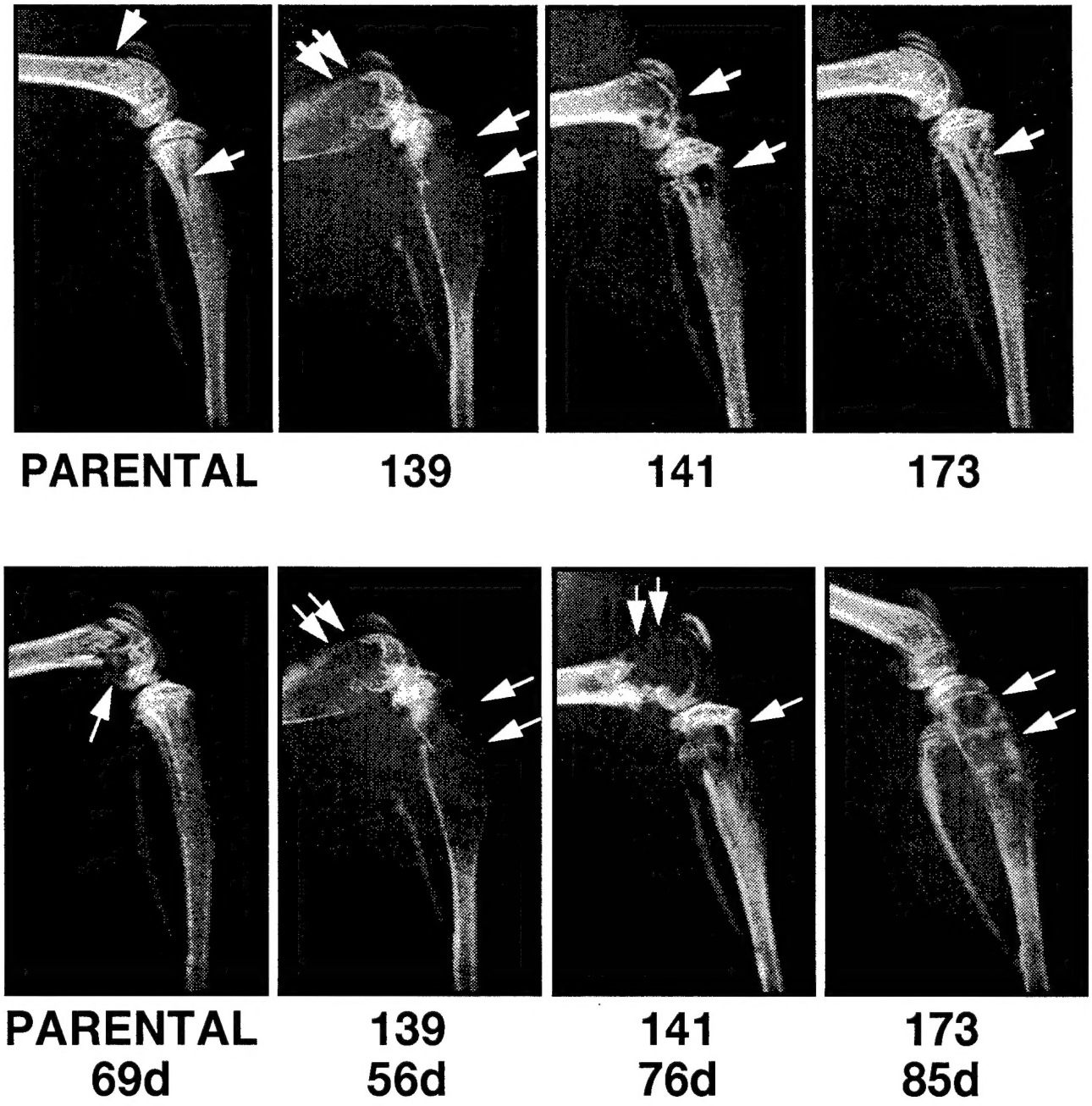
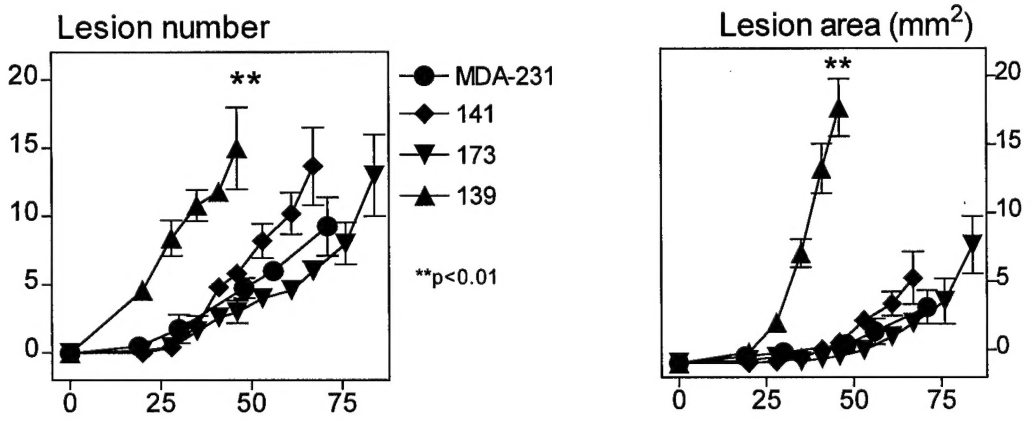


FIGURE 3: Representative radiographs of hindlimbs from mice bearing parental, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Top Panel: Radiographs were taken 48 days after inoculation of tumor cells. Osteolytic lesions are indicated by the arrows. The most bone destruction is evident in the mice bearing MDA/PTHrP-(1-139). Bottom Panel: Radiographs were taken at the time of sacrifice as indicated. All groups overexpressing PTHrP, regardless of the isoform, demonstrated significant bone destruction at the time of sacrifice compared with mice bearing the parental MDA-MB-231 tumors. However, this bone destruction was earlier and more severe in the mice bearing the MDA/PTHrP-(1-139) tumors. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 4



TIME (days since tumor inoculation)

FIGURE 4: Osteolytic lesion number and area on radiographs from mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors as assessed by computerized image analysis. Respective tumor cells were inoculated on day 0. Lesion number and area was measured from long bones of fore- and hind limbs. Values represent the mean ± SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 5

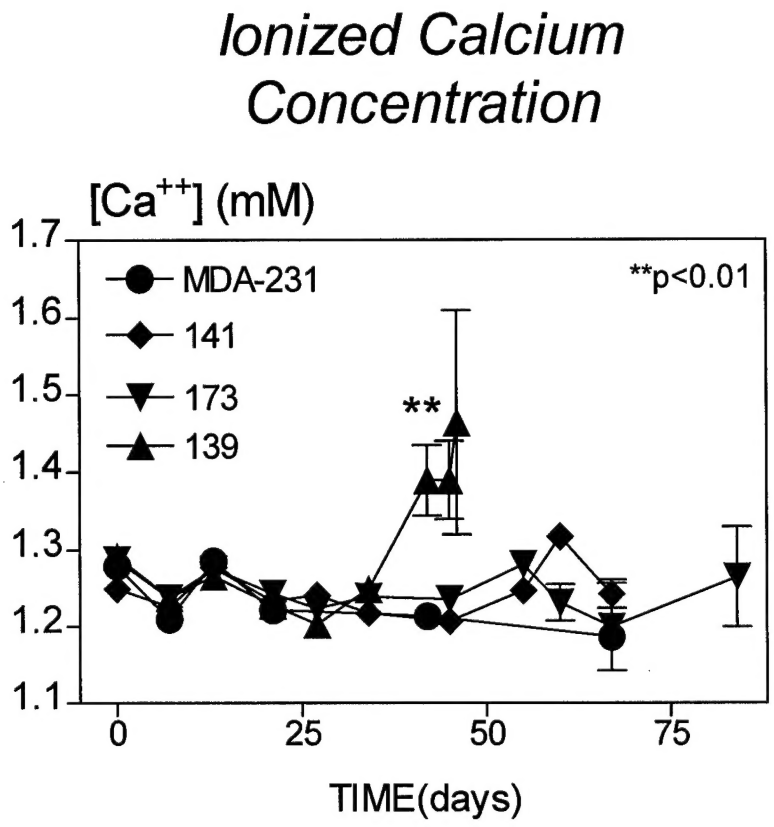


FIGURE 5: Whole blood ionized calcium concentrations in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Calcium concentrations were significantly higher in mice bearing the MDA/PTHrP-(1-139) tumors compared with the other groups. Values represent the mean ± SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.

FIGURE 6

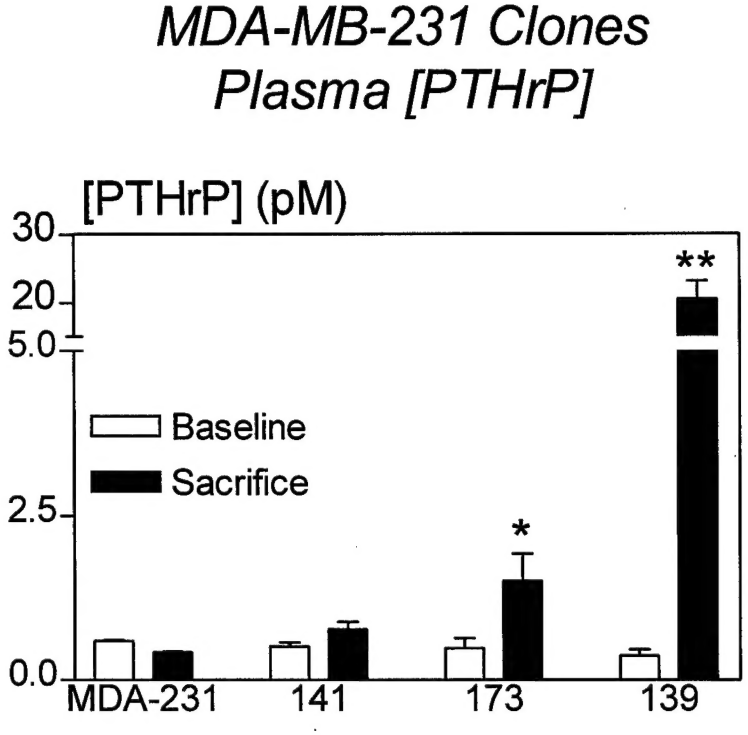


FIGURE 6: Plasma PTHrP concentrations in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-141), MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Plasma PTHrP concentrations at sacrifice were significantly higher than respective concentrations prior to tumor inoculation (baseline) in mice bearing MDA/PTHrP-(1-173) and MDA/PTHrP-(1-139) tumors. Values represent the mean ± SEM. N = 7 per group. Parental represent the untransfected and uncloned MDA-MB-231 cells while the representative transfectants are abbreviated with the respective isoform.